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2. That I am well acquainted with the French and English languages.

3. That the attached is, to the best of my knowledge and belief, a true translation into the English language of the accompanying copy of the specification filed with the application for a patent at the EPO on 9 November 1998 under the number 98203742.6 and the official certificate attached hereto.

4. That I believe that all statements made herein of my own knowledge are true and that all statements made on information and belief are true: and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application in the United States of America or any patent issuing thereon.

For and on behalf of RWS Group plc

The 12th day of April 2001

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

98203742.6

Der Präsident des Europäischen Patentamts;
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Coffee mannanase

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Bemerkungen
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Remarques

For the initial title see page 1 of the description.

Coffee mannanase

The present invention relates to the use of fragments of coffee DNA encoding at least one enzyme involved in the hydrolysis of polysaccharides consisting at least of simple or branched mannan molecules linked to each other via a β $(1\rightarrow4)$ linkage.

STATE OF THE ART:

Polysaccharides which contain mannose are frequently present in the cell walls of higher plants, in particular in leguminous plants, and are considered to be a carbohydrate store in the seeds.

In several plants, it has been shown that endo-β-mannanase activity is mainly detected in the endosperm of seeds undergoing germination (Bewley, Trends Plant Sci 2, 464-469, 1997).

In the coffee bean, galactomannans in particular are found. The latter represent approximately 24% of the dry weight of the bean (Bradbury and Halliday, J Agric Food Chem 38, 389-392, 1990). These polysaccharides consist of a linear chain of mannosyl residues which are linked to each other via β-$1\rightarrow4$ type linkages and to which are attached α-galactosyl residue monomers. It is also known that the enzyme named endo-β-mannanase (E.C 3.2.1.78) is a hydrolase which degrades $(1\rightarrow4)$-β-mannan polymers, thus facilitating the exit of the rootlet during germination and releasing small oligosaccharides which are then used as a source of energy for the growth of the young plant.

In industrial processes, during the treatment of coffee, the mannan molecules and their derivatives constitute a considerable portion of the insoluble sediments. In addition, the fraction of these molecules which dissolves during the first extraction (approximately 50%) is also very poorly soluble, and is therefore responsible for the majority of the secondary

precipitations which occur during the subsequent steps. In patent EP 0676145A1, therefore, it has been demonstrated that it is possible to hydrolyse coffee galactomannans using an immobilized mannanase extracted from *Aspergillus niger*.

Thus no gene or group of genes encoding at least one enzyme derived from coffee, which enzyme is involved in the hydrolysis of polysaccharides consisting at least of simple or branched mannan molecules linked to each other via a β $(1 \rightarrow 4)$ linkage has so far been identified and/or sequenced.

It would, therefore, be advantageous to isolate such enzymes derived from the coffee bean.

SUMMARY OF THE INVENTION:

The object of the invention is therefore to provide novel means for controlling, modifying and/or restoring the hydrolysis of coffee polysaccharides consisting at least of simple or branched mannan molecules linked to each other via a β $(1 \rightarrow 4)$ linkage.

To this effect, the present invention relates to any fragment of DNA derived from coffee encoding at least one enzyme involved in the hydrolysis of such polysaccharides.

The present invention also relates to the use of all or part of such fragments of DNA as a primer for carrying out a PCR or as a probe for detecting, *in vitro*, or modifying, *in vivo*, at least one coffee gene encoding at least one endo-β-mannanase.

The present invention also relates to any protein derived from the coffee bean, which is encoded by a coffee gene and involved in the hydrolysis of polysaccharides consisting at least of simple or branched mannan molecules linked to each other via a β $(1 \rightarrow 4)$ linkage, and which has the amino acid sequence SEQ ID NO: 2 or any amino acid sequence homologous to the latter.

Another subject of the invention relates to any microorganism and any plant cell comprising, integrated into its genome or by means of a plasmid which can replicate, a fragment of DNA according to the present invention.

Finally, the invention relates to a dietary, cosmetic or pharmaceutical composition comprising a fragment of DNA or a protein according to the invention.

DESCRIPTION OF THE FIGURES:

Figure 1 represents the 3.58 kb plasmid pMAN1.

Figure 2 represents the 2.98 kb plasmid pMAN2.

Figure 3 represents the 3.78 kb plasmid pMAN3.

Figure 4 represents the 4.56 kb plasmid pMAN4.

DETAILED DESCRIPTION OF THE INVENTION:

For the purposes of the present invention, the term "homologous sequence" is intended to mean any nucleic acid or amino acid sequence having an identical function, which differs from the sequences according to the invention only by the substitution, deletion or addition of a small number of nucleic acid bases or of amino acids, for example 1 to 500 base pairs (bp) or 1 to 150 amino acids.

In this context, two DNA sequences which, because of the degeneracy of the genetic code, encode the same polypeptide will in particular be considered to be homologous. Similarly, two functional proteins which are recognized by the same antibody, the ratio of the values of intensity of recognition of the two proteins by the antibody not exceeding 100, for example, will be considered to be homologous.

Also considered to be a homologous sequence will be that sequence which has more than 70% homology with the sequences according to the invention, in particular more than 80% or 90%. In the latter case,

the homology is determined by the ratio between the number of bases or of amino acids of a homologous sequence which are identical to those of a sequence according to the invention, and the total number of bases or of amino acids of said sequence according to the invention.

For the purposes of the present invention, the term "fragment which hybridizes" is intended to mean any fragment capable of hybridizing to the fragments according to the invention by the method of Southern Blot (Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, USA, 1989, chapters 9.31 to 9.58). Preferably, the hybridization is carried out under stringent conditions so as to avoid aspecific or relatively unstable hybridizations.

Finally, the term "fragment" or "fragment of DNA" should be understood to be a double-stranded DNA of chromosomal origin which can be synthesized, reproduced *in vitro* for example by the known method termed "Polymerase Chain Reaction", or reproduced *in vivo* in a bacterium of the *Escherichia coli* type, for example.

In the remainder of the description, the sequences SEQ ID NO: refer to the sequences given in the sequence listing hereinafter. The synthetic oligonucleotides SEQ ID NO: 3 to SEQ ID NO: 7 mentioned in the description and given in the sequence listing hereinafter are provided by Eurogentec (Parc Scientifique du Sart Tilman [Sart Tilman Scientific Park]-4102 Seraing-Belgium).

It has been possible to characterize a 1613 bp DNA sequence derived from coffee. Thus, the present invention relates to any fragments of DNA derived from coffee encoding at least one enzyme involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1$\rightarrow$4) linkage.

The fragment of DNA derived from coffee according to the invention preferably encodes at least one endo-β-mannanase.

It has been possible to show that all or part of the sequence SEQ ID NO: 1 makes it possible, subsequent to a transformation, to hydrolyse polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage in a host cell, such as a plant cell or a microorganism.

Taking into account the advantage of the present invention, the invention relates to any fragment of DNA having the nucleic acid sequence SEQ ID NO: 1 or any fragment of DNA which is homologous to or hybridizes to this nucleic acid sequence. Preferably, the invention relates to the fragment of DNA delimited by nucleotides 11 to 1292 of the nucleic acid sequence SEQ ID NO: 1.

Thus, the invention also relates to the novel enzymes encoded by the genes of the sequence SEQ ID NO: 1, in particular the sequences which are homologous to them. It is thus possible to envisage using them to modify or degrade such polysaccarides *in vitro*, for example. For this, it is preferable to purify at least one of these enzymes, by conventionally over-expressing their gene in a bacterium and isolating them conventionally, by precipitation and/or chromatography of the culture medium, for example.

The invention also relates to the use of all or part of fragments of DNA. Use can be made, in particular, of all or part of fragments of DNA according to the invention, of at least 10bp as a primer for carrying out a PCR or as a probe for detecting, *in vitro*, or modifying, *in vivo*, at least one coffee gene encoding at least one endo-β-mannanase.

Moreover, a subject of the present invention is also a protein derived from the coffee bean, which is

encoded by a coffee gene and involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage, and which has the amino sequence SEQ ID NO: 2 or any amino acid sequence homologous to the latter.

Another subject of the present invention relates to process for hydrolysing polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage, in which (1) a fragment of DNA encoding the enzymes according to the invention is cloned into a vector, said vector also comprising a sequence allowing autonomous replication or integration in a host cell, (2) a host cell is transformed with said vector, and then (3) the transformed host cell is cultured under conditions suitable for the hydrolysis of such polysaccharides.

The present invention therefore opens up the possibility of using fragments of DNA according to the invention to modify the production of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage in a host cell, in particular a coffee bean cell. It is thus possible to envisage expressing or over-expressing DNAs according to the invention, in a coffee bean cell, in order to produce such polypeptides intended to modify the aroma and the structure of the coffee beans, for example.

The present invention also makes it possible to have novel means for identifying coffee genes involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage.

Finally, the present invention also provides novel enzymes involved in the hydrolysis of such polysaccharides. These enzymes can thus be

advantageously used to hydrolyse or modify such polysaccharides, *in vitro*.

The present invention also relates to a plant cell comprising, integrated into its genome or by means of a recombinant vector, a fragment of DNA encoding at least one enzyme involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage.

Preferably, this plant cell comprises a fragment of DNA having the nucleotide sequence SEQ ID NO: 1, or a fragment of DNA having a nucleic acid sequence which is homologous to or hybridizes to the nucleic acid sequence SEQ ID NO: 1, or a fragment of DNA comprising at least nucleotides 11 to 1292 of the nucleic acid sequence SEQ ID NO: 1.

Preferably, this plant cell is a coffee cell. It is possible in particular to choose, as coffee cells, cells derived from a plant of Coffea canephora var. robusta, Coffea arabica or any other species of the Coffea genus.

The present invention also relates to any plant or any seed consisting of plant cells comprising, integrated into its genome or by means of a recombinant vector, a fragment of DNA encoding at least one enzyme involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage.

Any microorganism comprising, integrated into its genome or by means of a plasmid which can replicate, a fragment of DNA according to the invention such that it expresses at least one enzyme involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage is also a subject of the present invention.

Another subject of the invention relates to any dietary, cosmetic or pharmaceutical composition comprising a fragment of DNA according to the invention or a protein according to the invention.

Finally, the present invention relates to a process for treating coffee beans, in which all or part of the protein according to the invention is used. It is in particular possible to use all or part of the protein according to the invention to increase the percentage of solids extracted, during the treatment of coffee beans. Using all or part of the protein according to the invention, it is thus possible to increase the extraction yield while at the same time decreasing the amount of sediment.

After over-expression of the fragment of DNA according to the invention in a microorganism, in a fungus or in an undifferentiated plant cell, the sediments can be treated with the more or less purified enzyme, so as to thus increase the extraction yields.

After over-expression of the fragment of DNA according to the invention in a microorganism, in a fungus or in an undifferentiated plant cell, it is also possible to treat the coffee liquor, so as to decrease the sedimentation due to the mannans which gel.

The present invention is described in more detail hereinafter with the aid of the further description which will follow and which refers to examples of production of fragments of DNA, of recombinant plasmids and of transformed bacteria according to the invention. It goes without saying, however, that these examples are given by way of illustration of the subject of the invention for which they in no way constitute a limitation. The handling of the DNA, the cloning and the transformation of bacterial cells are, in the absence of instructions to the contrary, carried out according to the protocols described in the manual by Sambrook *et al.*, mentioned

above. The percentages are given by weight, unless otherwise indicated.

Test 1: Isolation of the coffee endo-β-mannanase cDNA

1. <u>Isolation of the total RNAs and of the poly A+ messenger RNAs from the germinating coffee bean</u>

Coffee beans (*Coffea arabica* var. 2308) are harvested at the mature stage, depulped immediately and dried for three days at room temperature. Next, the parchment skin of the beans is removed, and they are dried and then sterilized in order to be germinated in culture *in vitro*. To do this, they are placed in Rovral (0.12% v/v) for 1 hour, rinsed with sterile water, placed in a solution of calcium hypochlorite (6% w/v) to which a few drops of Teepol emulsifier are added, for 1 hour, and then rinsed 4 times with sterile water before being cultured in test-tubes on an agar-water medium. The germination occurs at 25°C in the presence of light. The moment when the beans are placed on the agar bed is considered to be day after soaking zero (DAS = 0).

The total RNAs of beans are extracted after 22 days of germination (DAS 22).

To do this, the bean is rapidly ground in liquid nitrogen and the powder obtained is resuspended in 8 ml of buffer at pH 8 containing 100 mM Tris HCl, 0.1% w/v of SDS and 0.5% v/v of β-mercaptoethanol, it is homogenized with one volume of phenol saturated with 100 mM Tris HCl, pH 8, and then it is centrifuged at 12 000 *g* for 10 min at 4°C, so as to extract the aqueous phase, which is centrifuged (i) once with an equivalent volume of phenol, (ii) twice with an equivalent volume of phenol:chloroform (1:1) and (iii) twice with an equivalent volume of chloroform.

The total nucleic acids are then precipitated for 1 h at -20°C by adding to the aqueous phase 1/10 of

a volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of ethanol.

The resulting mixture is then centrifuged at 12 000 *g* for 30 min at 4°C, and the pellet is taken up in 10 ml of H_2O, before re-precipitating the nucleic acids in the presence of LiCl (2 M final) and of ethanol (2.5 volumes).

After centrifugation, the pellet of total RNAs is taken up in 1 ml of H_2O and it is digested for 1 h at 37°C with RQ1 DNAse (Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin 53711 USA) in order to eliminate any trace of DNA, and then the total RNAs are deproteinated by treating with phenol and with chloroform, before precipitating them in the presence of sodium acetate as described above.

The total RNAs are then taken up in 500 µl of H_2O, and they are quantified by spectrophotometric assay at 260 nm. Their quality is analysed by agarose gel electrophoresis in the presence of formaldehyde.

To do this, the poly A+ messenger RNAs (mRNAs) are then purified from 500 µg of total RNAs using the Oligotex-dT purification system (Qiagen INC., 9600 De Soto Avenue, Chatsworth, California, 91311 USA), and then the quantity of mRNAs is evaluated using the DNA Dipstick kit (InVitrogen BC, De Schelp 12, 9351 NV Leek, the Netherlands).

2. Construction and screening of the cDNA library

The synthesis of cDNA, required for constructing the libraries, is carried out according to the recommendations supplied in the "Riboclone cDNA synthesis system M-MLV (H-)" kit (Promega, USA), except for the *Eco*RI linker ligation step. This makes it possible to clone these cDNAs directly into the vector pCR-Script SK(+) (Stratagene, 11011 North Torrey Pines Road, La Jolla, California 92037, USA). The efficiency of this cDNA synthesis reaction is monitored by adding

alpha-(^{32}P)-dCTP during the synthesis of the two DNA strands.

After migration on alkaline agarose gel (Sambrook *et al.,* 1989), the length of the neosynthesized cDNAs is estimated as ranging from 0.2 to more than 4.3 kb. The quantifications, using the DNA Dipstick kit (InVitrogen BV, De Schelp 12, 93 51 NV Leek, the Netherlands), show that approximately 100 ng of cDNA are synthesized from 1 μg of mRNA.

The cDNAs ligated into the vector pCR-Script SK(+) (Stratagene, USA) were used to transform the *E. coli* strain XL2-Blue MRF' (Stratagene, USA). The bacteria which contain recombinant vectors are selected on plates of LB (Luria-Bertani) medium containing 20 $\mu g.ml^{-1}$ of ampicillin, and 80 $\mu g.ml^{-1}$ of methicillin, and in the presence of IPTG and of X-Gal (Sambrook *et al.,* 1989). They are then cultured on petri dishes, so as to obtain approximately 300 clones per dish. These clones are transferred onto a Nylon filter and they are then treated according to the recommendations provided by Boehringer Mannheim (Boehringer Mannheim GmbH, Biochemica, Postfach 310120, Mannheim 31, DE). The plasmids of this cDNA library are also extracted from an overnight culture of these transformants, in the presence of 25 ml of LB medium containing 50 $\mu g.ml^{-1}$ of ampicillin, and using the "QiaFilter Plasmid MidiKit" (Qiagen INC., USA).

3. Isolation of the cDNA encoding the coffee endo-β-mannanase

In a preliminary experiment, the synthesis of the first strand of cDNA is carried out according to the recommendation supplied in the "Riboclone cDNA synthesis system M-MLV (H-)" kit (Promega, USA). The efficiency of this reaction is monitored by adding alpha-(^{32}P)-dCTP during the cDNA synthesis.

The synthesis of the second strand of the cDNA is then carried out by performing an RT (Reverse Transcription)-PCR reaction (US Patent 4,683,195 and US Patent 4,683,202) using the synthetic oligonucleotide MAN 2, having the nucleic acid sequence SEQ ID NO: 3, and the synthetic oligonucleotide DT15, having the nucleic acid sequence SEQ ID NO: 4. The synthetic oligonucleotide MAN 2 corresponds to the amino acid sequence situated between amino acids 206 and 212 of the protein sequence of *Lucopersicon esculentum* (Bewley et al., Planta 203, 454-459, 1997) which is also conserved in the endo-β-mannanase protein sequences of *Trichoderma reesei* (Stalbrand et al., GenBank Accession Number L25310, 1993) and *Aspergillus aculeatus* (Christgau et al., Biochem Mol Biol Internat 33, 917-925, 1994).

The PCR reaction is carried out in the presence of 1 to 10 ng of first strand of cDNA, in a final volume of 50 µl containing 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.1 $mg.ml^{-1}$ gelatin, 0.2 mM of each dNTP, 0.25 µM of each oligonucleotide (MAN 2 and DT15) and 3 units of *Taq* DNA polymerase (Stratagene, USA). The reaction mixture is covered with 50 µl of mineral oil and incubated for 35 cycles (94°C - 30 s, 45°C - 30 s, 72°C - 3 min) followed by a final extension at 72°C for 7 min. At the end of this reaction, a majority PCR product approximately 600 bp long is obtained, which was purified on electrophoresis gel using the "Gel Nebulizer - Micropure 0.22 µm" DNA extraction system (Amicon INC., 72 Cherry Hill Drive, Beverly, Massachusetts 01915 USA). This fragment is treated with *Pfu* DNA polymerase (Stratagene, USA) in order to convert its sticky ends to blunt ends. This reaction takes place in a final volume of 13 µl containing approximately 200 ng of DNA fragment, 1mM of dNTP, 10mM KCl, 6mM $(NH_4)_2SO4$, 20 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 2 mM $MgCl_2$, 10 µg ml^{-1} BSA and 2.5

units of *Pfu* DNA polymerase. The reaction mixture is covered with 20 μl of oil, and this is incubated at 72°C for 30 min. The mixture obtained is desalified on a Microcon 50 cartridge (Amicon INC., USA) and ligated into the vector pCR-Script SK(+).

For this, 50 ng of PCR fragment are added to a ligation mixture which comprises 10 mM KCl, 6 mM $(NH_4)_2SO4$, 20 mM Tris-HCl, pH 8, 0.1% Triton X-100, 2 mM $MgCl_2$, 10 μg/ml BSA, 10 ng of the cloning vector pCR-Script SK(+), 5 units of the *Sfr*I restriction enzyme, 4 units of T4 DNA ligase and 5 mM of rATP. This reaction is incubated for 60 min. at 25°C and is then used to transform the *E. coli* strain XL2-Blue MRF' (Stratagene, USA). The bacteria which contain recombinant vectors are selected on plates of LB medium containing 20 μg.ml^{-1} of ampicillin, and 80 μg.m^{-1} [sic] of methicillin, and in the presence of IPTG and of X-Gal (Sambrook *et al.*, 1989).

At the end of the transformation, a clone was isolated which harbours the recombinant plasmid pMAN1, described in Figure 1 hereinafter. This vector contains the PCR fragment obtained above which is cloned into the *Sfr*I site of the vector pCR-Script (SK+). This cDNA was sequenced according to the "T7 sequencing kit" protocol Pharmacia Biotech AB, (Björkgatan 30, 75184 Uppsala, Sweden), in the presence of alpha (^{35}S)-dATP. The analysis of its sequence shows that it is located between nucleotides 743 and 1369 of the sequence SEQ ID NO: 1 and is bordered, at its 5' and 3' ends, by the respective sequences homologous to the oligonucleotide MAN2. By comparing with the protein sequence of *Trichoderma reesei* and *Aspergillus aculeatus* mannanase, it is deduced that the cDNA cloned in this way corresponds to a partial cDNA of the coffee endo-β-mannanase.

In order to isolate the full-length cDNA of the coffee endo-β-mannanase, a hybridization was carried

out on colonies by testing 1800 transformants of *E. coli* XL 2-Blue MRF' from the cDNA library, which hybridation was carried out on coffee beans undergoing germination.

The transformants are transferred onto a Hybond N+ nylon filter (Amersham International plc., Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, UK) according to the suppliers' recommendations, and analysed by molecular hybridization with the MAN1 probe (50 ng). This probe is obtained after digesting the vector pMAN1 with the *Sma*I restriction enzyme. It was purified on electrophoresis gel and labelled by random primer extension with 50 μCi of alpha-(^{32}P)-dCTP according to the protocol of the Megaprime kit (Amersham, UK). After hybridization, washing and autoradiography of the filters, a positive clone is detected which harbours the recombinant vector pMAN2, described in Figure 2 hereinafter. This vector contains an approximately 1000 bp fragment of DNA, which was sequenced on both strands (Eurogentec Bel s.a.-Parc Scientifique du Sart Tilman [Sart Tilman Scientific Park] - 4102 Seraing-Belgium). It comprises, in fact, the last 1022 base pairs of the sequence SEQ ID NO: 1, but again constitutes only a partial cDNA of the coffee endo-β-mannanase.

In order to isolate the 5' end of the coffee mannanase cDNA, a PCR reaction was carried out according to the conditions described above, but with the exception of the following parameters. 20 ng of the plasmid DNA library (22 days of germination), the synthetic oligonucleotide MAN60, corresponding to the sequence SEQ ID NO. 5, and the universal oligonucleotides ForM13 and RevM13, which correspond to the sequences SEQ ID NO: 6 and SEQ ID NO: 7, respectively, were used. These primers are each located at approximately 100 bp on both sides of the *Sfr*I cloning site of the vector pCR-Script SK (+). The

primer MAN60 is, itself, located between nucleotides 803 and 819 of the sequence SEQ ID NO: 1. After this reaction, an approximately 900 bp amplification fragment [MAN60/ForM13] was obtained, which was digested with the *Sma*I restriction enzyme in order to eliminate the sequences of the plasmid pCR-Script SK (+). This digested DNA was ligated into the vector pCR-Script SK (+) so as to give the vector pMAN3 described in Figure 3 hereinafter. The analysis of its sequence reveals that it corresponds to the 5′ end of the cDNA encoding the coffee mannanase and that it is located between nucleotides 1 and 819 of the sequence SEQ ID NO: 1.

Since these experiments did not allow us to isolate a full length cDNA encoding the coffee mannanase, we decided to re-screen the plasmid DNA library (22 days of germination), this time using the "ClonCapture cDNA Selection Kit" (Clontech Laboratories Inc., 1020 East Meadow Circle, Palo Alto California 94303-4230 USA). In this case, the MAN3 probe, which is obtained by double digestion of the plasmid pMAN3 with the *Bam*HI and *Sac*I restriction enzymes, is used. This probe is biotinylated according to the protocol defined by Clontech (USA), and it is used to enrich the cDNA library in plasmid containing all or part of the sequences of the cDNA encoding the coffee mannanase. This enriched library was amplified in *E. coli*, and was then screened using the MAN3 cDNA probe described above, which was labelled by random primer extension according to the protocol of the Megaprime kit (Amersham, UK).

At the end of this screen, one positive clone in particular was selected, which contained an approximately 1600 bp cDNA cloned into the vector pCR-Script SK (+). This recombinant plasmid is named pMAN4, described in Figure 4 hereinafter, and the cDNA which it harbours was sequenced. The analysis thereof in the

Genbank databank (release 106.0) (Genetics Computer Group Inc., University Research Park, 575 Science Drive, Madison, Wisconsin 53711 USA) showed that it corresponds to the entire sequence SEQ ID NO: 1.

4. Analysis of the full-length cDNA encoding the coffee endo-β-mannanase

The analysis of the nucleic acid sequence shows that this full-length cDNA contains a short 10 bp transcribed, untranslated sequence at its 5′ end, and a very long sequence (36 bp) corresponding to the polyA tail in the transcribed, untranslated 3′ end of 280 bp. Within the latter sequence, an absence of AATAAA motifs which are presumed to be involved in polyadenylation mechanisms is observed, but, however, the presence of several nucleic acid motifs rich in GT (position) which might fulfil this role (Morgan et al., Plant Cell 2: 1261-1272, 1190) is noted.

Also noted are the presence of two inverted repeat sequences, which are very conserved between each other, and the existence of two large 34 bp direct repeat sequences, at position 1383-1417 and 1440-1474, which contain several repeats of the nucleic acid motif AGT(C/A)A(T/A)(G/A). The precise functions of these sequences are unknown, but it may be presumed that they are involved in mechanisms of stability of the mRNAs or of efficiency of translation, for example (Gallie, Plant Mol Biol, 32, 145-158, 1996).

The sequence SEQ ID NO: 1 contains an open reading frame of 428 codons, which begins with the ATG codon at position 11 and ends with a TGA codon at position 1292. The protein deduced from this cDNA has an approximate molecular weight of 48349 Da and has a very hydrophobic protein segment which corresponds to the first 30 amino acids of the sequence SEQ ID NO: 1. This protein sequence might correspond to a sequence of signal peptide type. In this case, the molecular weight

of the protein is expected to be less than or equal to 45000 Da in its mature form.

The existence of several potential glycosylation sites (Asn / X / Ser or Thr) is also noted. The first two are located in the potential signal peptide, at position 8 and 11 of the sequence SEQ ID NO: 2, and are therefore presumed to be absent in the mature form of the mannanase. The other two are located at the C-terminal position, at position 389 and 412 of the sequence SEQ ID NO: 2. One of these two potential sites must necessarily be glycosylated since coffee mannanase is effectively retained by filtration over a concanavalin A affinity column.

Test 2: Measurement of the peak of activity of endo-β-mannanase during germination

Beans of the variety *C. arabica* Caturra are harvested at the mature stage and treated as defined above during the isolation of the RNAs.

The batches of beans are harvested at various stages of germination (DAS 7, 14, etc), and then are ground in liquid nitrogen. Next, the powder is homogenized in a proportion of 1 g per 5 ml, in an extraction buffer (200/100 mM phosphate-citrate, pH 5, 10 mM meta-bisulphite, $Na_2S_2O_5$, 5 mM EDTA and one tablet/50 ml of "Complete" protease inhibitor [Cat. No. 1836 145, Boehringer Mannheim, Mannheim, Germany]), for 20 min at 4°C. The homogenate is then centrifuged at 12 000 g for 20 min at 4°C, and the supernatant recovered and centrifuged a second time. The supernatant, that is to say the crude enzymatic extract, is then aliquoted and placed at -80°C.

The activity of the endo-β-mannanase is assayed according to the following method. A 400 µl extract is added to a reaction buffer of acetic acid-sodium acetate (200 mM, pH5,100 mM NaCl) (1.6 ml) containing

insoluble substrate (AZCL-Galactomannan, Megazyme, Co. Wicklow, Ireland) in a final quantity of 1% w/v. The reaction commences by adding the extract, and takes place at 37°C with stirring. In order to calculate the initial slope of the reaction, a 400 µl aliquot of medium is removed every 15 min for 1 hour, heated at 100° for 5 min and then centrifuged at 12 000 g for 2 min. The supernatant is read at 590 nm. The specific activity is expressed in optical absorption units (AU).min^{-1}.mg $protein^{-1}$, after having assayed the protein concentration in each extract by the Bradford method (Bradford, Anal. Biochem. 72, 248-254, 1976). Thus, it is found that the activity is virtually zero during the first 14 DAS, and subsequently increases gradually up to a peak around 28 DAS. After 28 DAS, the activity slowly decreases.

Test 3: Endo-β-mannanase purification steps

According to the results described above, the purification strategy is continued using at least 640 ml (750 beans) of a 28-DAS crude enzyme extract having an activity of around 1.3 $AU.min^{-1}.mg\ protein^{-1} \times 10^{-3}$, a total protein content of approximately 640 mg and a total activity of 832 $AU.min^{-1}$ [lacuna].

1. Ammonium sulphate precipitation:

Initially the crude enzyme extract is fractionated by ammonium sulphate precipitation at 4°C. The ammonium sulphate is added slowly with stirring until a saturation level of 35% is obtained, and the solution is then centrifuged at 12 000 g at 4°C for 20 min. The precipitate thus obtained from 640 ml of crude enzymatic extract is taken up in 16 ml of extraction buffer, the protein concentration in this extract being around 3 $mg.ml^{-1}$ and the endo-β-mannanase activity around 13 $AU.min^{-1}.mg^{-1} \times 10^{-3}$, that is to say a 10-fold enrichment of the enzyme and a recovery of 624 $AU.min^{-1}$ [lacuna] of the total activity, i.e. 75%.

2. Separation by gel filtration:

Following the fractionation by precipitation in the presence of ammonium sulphate, the extract is desalted using a PD-10 G-25M (Pharmacia Biotech, Sweden). The elution is carried out with the extraction buffer supplemented with 150 mM NaCl (extraction buffer + NaCl). The entire sample is then fractionated on a Sephacryl S-200 HiPrep column (Pharmacia Biotech, Sweden), with a 120 ml volume, pre-equilibrated with the extraction buffer + NaCl and calibrated with molecular weight markers. The separation is carried out at 4°C with a flow rate of 0.46 $ml.min^{-1}$ and the experiment is repeated 4 times, each time using 4 ml of the extraction obtained by ammonium sulphate

precipitation. Under these conditions, for each separation, the endo-β-mannanase activity comes off in a total volume of 13 ml (in 1 ml fractions) with a retention volume of around 100 ml, which corresponds to a molecular weight of approximately 50 000 Da. The enrichment is, on average, 9-fold with respect to the sample injected onto the column, with a specific activity of around 117 $AU.min^{-1}.mg^{-1} \times 10^{-3}$, a total protein content of approximately 2.25 mg and an enzyme activity recovery with respect to the CEE of 31%.

The total protein in the 52 ml of solution is precipitated by treatment with ammonium sulphate at 80% saturation as described above, and the protein is taken up in 3 ml of a 20 mM Tris-HCl buffer, pH 8, containing the "Complete" protease inhibitor (Boehringer Mannheim, Germany), 1 $tablet.50\ ml^{-1}$. This solution is then desalted as described above, eluting the protein material with the same 20 mM Tris-HCl buffer, pH 8. The total protein is recovered in approximately 5 ml, with a concentration of approximately 0.4 $mg.ml^{-1}$ (2 mg total), an activity of 115 $AU.min^{-1}.mg^{-1} \times 10^{-3}$, a total activity of 230 $AU.min^{-1}$ [lacuna], an enzyme activity recovery with respect to the CEE of 27%, and an enrichment factor of 88. Alternatively, the protein material can be kept at 4°C in a solution of 20 mM Tris-HCl, pH 8, containing 2.5 M ammonium sulphate.

3. Separation by ion exchange:

Tests having been carried out beforehand to establish the titration, as a function of pH, of the endo-β-mannanase activity, a 5 ml DEAE Sepharose Fast Flow (Pharmacia Biotech, Sweden) stationary phase column is equilibrated with the 20 mM Tris-HCl buffer, pH 8, at 4°C. The desalted extract described above (5 ml) is loaded onto the top of the column and the separation takes place at 4°C without applying additional pressure. Once the sample has penetrated

into the column, the column is washed with 15 ml of the Tris-HCl buffer, pH 8. The proteins are then eluted with the same buffer containing 500 mM NaCl. The eluate coming off the column is fractionated in 1 ml cuvettes and controlled for the protein content at 280 nm, and the endo-β-mannanase activity is assayed. A total activity of 209 $AU.min^{-1}$ is recovered in 3 fractions, normally in fractions 4, 5 and 6, in a total volume of 3 ml, with a protein concentration of approximately 110 $\mu g.ml^{-1}$ and a specific activity of 639 $AU.min^{-1}.mg^{-1} \times 10^{-3}$. Thus, this sample is enriched for the endo-β-mannanase activity by a factor of 491 with respect to the CEE and represents an original total activity recovery of 24%.

4. Separation by Concanavalin A-affinity column:

This step takes place under the same conditions as the separation above. A column is prepared with 2 ml of Concanavalin A Sepharose (Pharmacia Biotech, Sweden) stationary phase, equilibrated with 20 ml of a 20 mM Tris-HCl, pH 7.4, 500 mM NaCl buffer. The sample described above (3 ml) is loaded onto the column, and then the column is washed with 10 ml of the equilibration buffer. The elution is carried out with the same buffer containing 300 mM methyl α-D-mannopyranoside (Sigma, St. Louis, MO, USA, cat. no. M-6882), and the eluate is fractionated in 1 ml cuvettes and controlled at 280 nm, and the endo-β-mannanase activity is assayed. The enriched endo-β-mannanase total activity is distributed over three 1 ml fractions, the whole having a mean protein concentration of around 1 $\mu g.ml^{-1}$, a specific activity of 3 500 $AU.min^{-1}.mg^{-1} \times 10^{-3}$, that is to say an enrichment of 2 690-fold, and a total activity of 105 $AU.min^{-1}$ [sic] which represents a total activity recovery with respect to the CEE of approximately 12% [sic].

Analysis of this fraction by SDS-PAGE reveals the presence of a major protein band with a molecular weight of between 45 000 and 50 000 Da.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: SOCIETE DET PRODUITS NESTLE S.A

(B) STREET: AVENUE NESTLE 55,

(C) CITY: VEVEY

(D) STATE OR PROVINCE: VAUD

(E) COUNTRY: SWITZERLAND

(F) POST CODE: 1800

(G) TELEPHONE: 021 924 34 20

(H) FAX: 021 924 28 80

(ii) TITLE OF THE INVENTION: COFFEE MANNANASE

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1613 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CGATGGTGGC GATCGAGCTC TTCAAATGTC CCCCGGAGTC TATGATGAAC GTGTCTTTCA    360
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CGGAGTACAA GTGAATAGCG ATGATGATTT TTACACCAAG AATGCTGTCA AGGGATATTA 540
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CTCCGGAAAA ACCTTAAATG CTTGGGTTCA AGAAATGGCA ACTTACGTCA AATCACTCGA 720
TAACAAACAC CTTCTAGAAA TAGGCATGGA GGGATTCTAC GGAGATTCAA TGCCAGGCAA 780
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 427 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: [lacuna]

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Ser Ser Ala Ser Arg Phe Ile Gln Thr Arg Gly Thr Arg Phe Val Leu
35 40 45

Gly Gly Tyr Pro Phe Phe Phe Asn Gly Phe Asn Ser Tyr Trp Met Met
50 55 60

His Val Ala Ala Glu Pro Ser Glu Arg His Lys Ile Ser Asn Val Phe
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Arg Glu Ala Ala Ala Thr Gly Leu Thr Val Cys Arg Thr Trp Ala Phe
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Ser Asp Gly Gly Asp Arg Ala Leu Gln Met Ser Pro Gly Val Tyr Asp
100 105 110

Glu Arg Val Phe Gln Ala Leu Asp Phe Val Val Ser Glu Ala Arg Lys
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Gly Gly Arg Thr Gln Tyr Val Thr Trp Ala Lys Asn Ala Gly Val Gln
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Lys Lys Gln Tyr Asn Pro Gly Tyr Gln Val Gly Thr Asp Phe Ile Thr
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Lys Pro Leu Val Leu Ala Glu Phe Gly Lys Ser Ser Lys Asp Pro Gly
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Val Leu Ser Gln Asn Pro Ser Thr Gly Arg Ile Ile Ser Gln Gln Ser
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Arg Gln Met Thr Ser Leu Asp His Met Ser Ser Asn Arg Thr Asn Ser
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "SYNTHETIC OLIGONUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGNATGGARG GNTTYTAYGG 20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "SYNTHETIC OLIGONUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc - "SYNTHETIC NUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AAATCTGTGC CCACTTG 17

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc - "SYNTHETIC NUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GTAAAACGAC GGCCAGT 17

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc - "SYNTHETIC NUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CAGGAAACAG CTATGAC 17

Claims

1. Fragment of DNA derived from coffee encoding at least one enzyme involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage.

2. Fragment of DNA according to Claim 1, encoding at least one endo-β-mannanase.

3. Fragment of DNA according to Claims 1 and 2, having the nucleic acid sequence SEQ ID NO: 1.

4. Fragment of DNA according to Claim 3, comprising at least nucleotides 11 to 1292 of the nucleic acid sequence SEQ ID NO: 1.

5. Fragment of DNA which is homologous to or hybridizes to a fragment of DNA according to either of Claims 3 and 4.

6. Recombinant vector comprising a fragment of DNA according to one of Claims 3 to 5.

7. Use of all or part of fragments of DNA according to Claims 3 to 5, of at least 10 bp, as a primer for carrying out a PCR or as a probe for detecting, *in vitro*, or modifying, *in vivo*, at least one coffee gene encoding at least one endo-β-mannanase.

8. Protein derived from the coffee bean, which is encoded by a coffee gene and involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage, and which has the amino acid sequence SEQ ID NO: 2 or any amino acid sequence homologous to the latter.

9. Plant cell comprising, integrated into its genome or by means of a recombinant vector, a fragment of DNA encoding at least one enzyme involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β (1→4) linkage.

10. Plant cell according to Claim 9, comprising a fragment of DNA according to one of Claims 3 to 5.

11. Plant cell according to Claims 9 and 10, characterized in that it is a coffee cell.

12. Plant or seed consisting of plant cells according to one of Claims 9 to 11.

13. Microorganism comprising, integrated into its genome or by means of a plasmid which can replicate, a fragment of DNA according to one of Claims 3 to 5 such that it expresses at least one enzyme involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β $(1\rightarrow4)$ linkage.

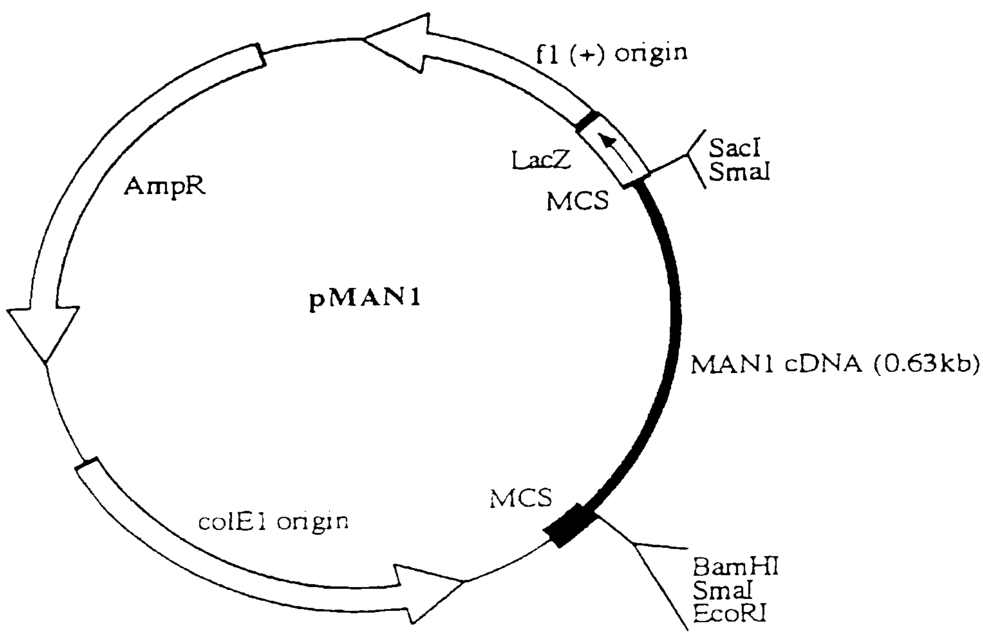
14. Dietary, cosmetic or pharmaceutical composition comprising a fragment of DNA according to Claims 1 to 5 or a protein according to Claim 8.

15. Process for treating coffee beans, in which all or part of the protein according to Claim 8 is used.

Abstract

Coffee mannanase

Fragment of DNA derived from coffee encoding at least one enzyme involved in the hydrolysis of polysaccharides consisting at least of pure or branched mannan molecules linked to each other via a β $(1\rightarrow4)$ linkage.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EcoRI

Fig 1/4

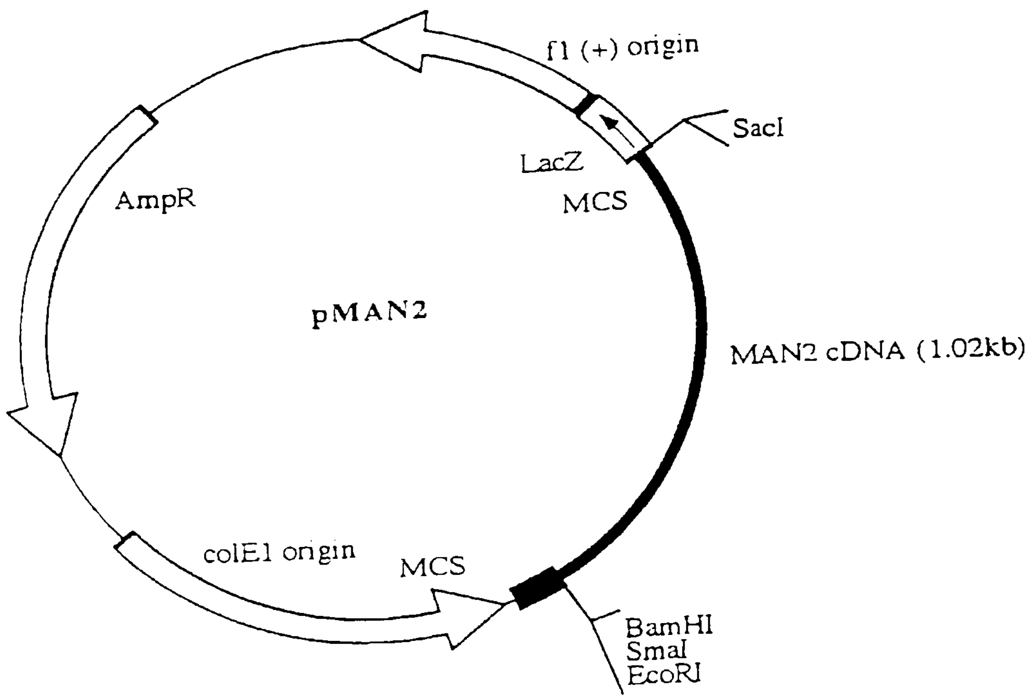
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Fig 2/4

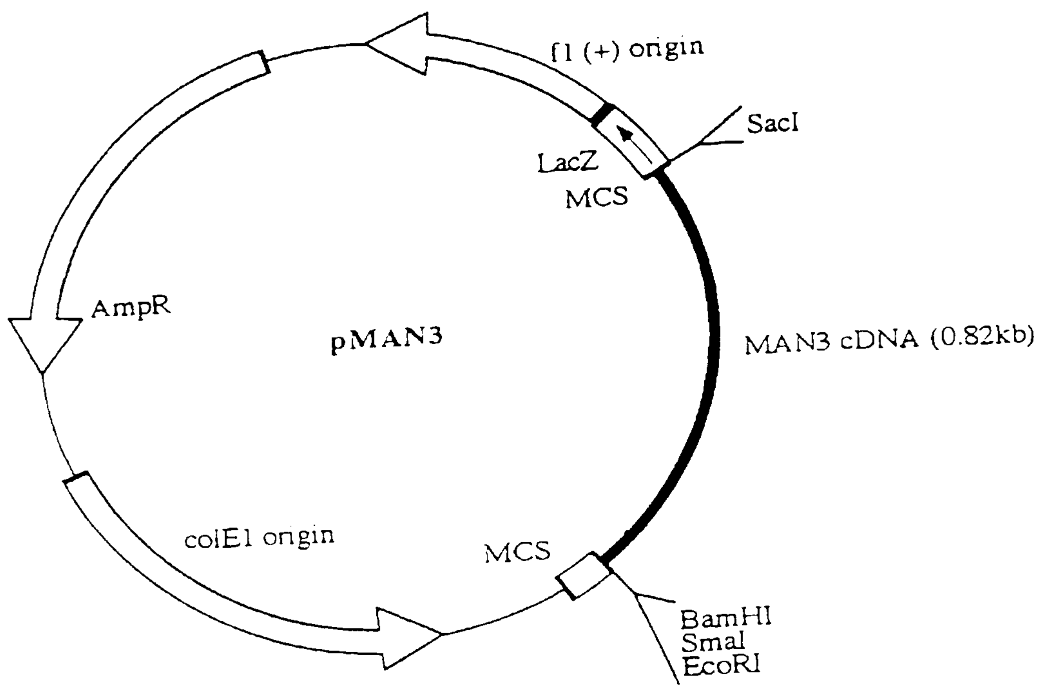
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Fig 3/4

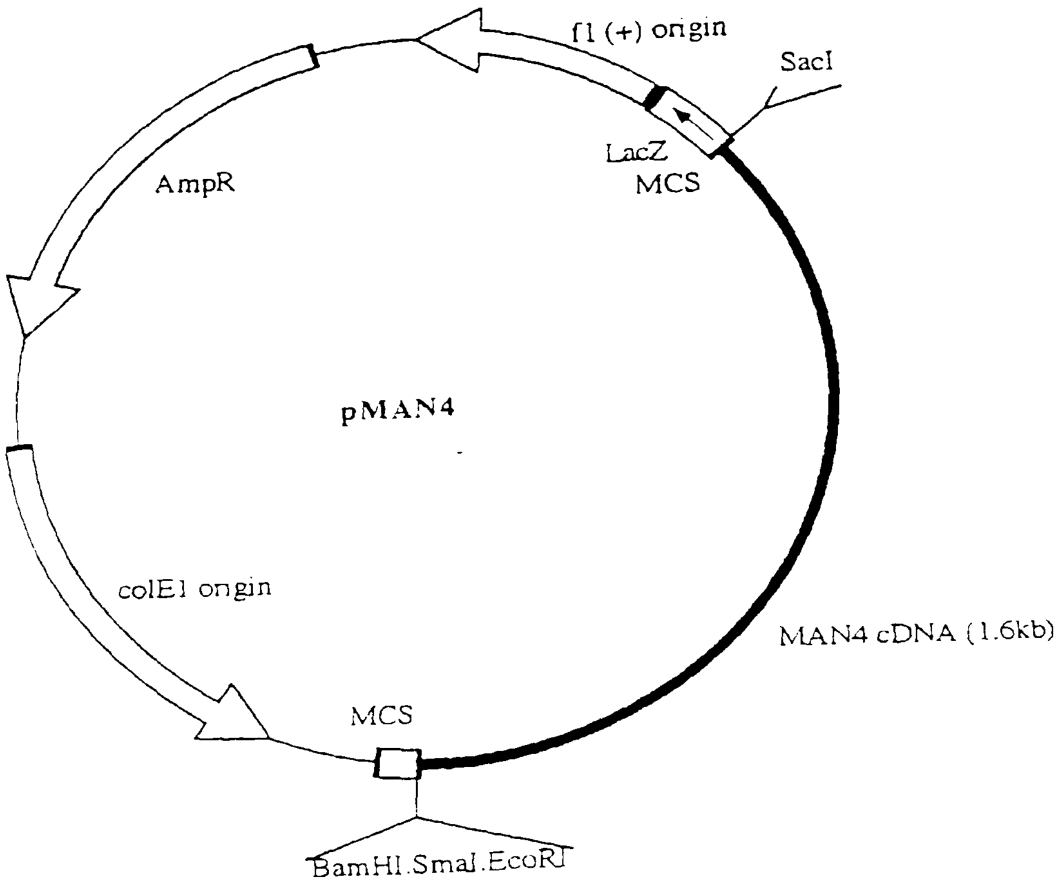
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Fig 4/4